SUPPLEMENTAL PROTOCOL

Multiple Immunofluorescent Labeling Using Two or More Mouse Monoclonal Primary Antibodies

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1. **Preparation of tissue.** Fix sections with the appropriate fixative for the antigen under study *(Please see Note 1)*.
2. **Air dry sections.**
3. Wash sections 2 x 2 min in buffer (PBS).
4. **Avidin/biotin blocking step.** Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 min. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 min. Wash sections 2 x 2 min in buffer. This blocking step may be eliminated if suitable controls have determined this step to be unnecessary.
5. **Mouse Ig blocking step.** Incubate sections for 1 h in working solution of M.O.M.® Mouse Ig Blocking Reagent *(Please see Note 2)*.
6. Wash sections 2 x 2 min in buffer *(Please see Note 2)*.
7. **Protein blocking step.** Incubate tissue sections for 5 min in working solution of M.O.M. diluent.
8. **Primary antibody.** Tip off excess M.O.M. diluent from sections. Dilute primary antibody in M.O.M. diluent to the appropriate concentration. Incubate section in diluted primary antibody for 30 min *(Please see Note 3)*.
9. Wash sections 2 x 2 min in buffer.
10. **Secondary antibody.** Apply working solution of M.O.M. Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 min.
11. Wash sections 2 x 2 min in buffer.
12. **Avidin conjugate.** Apply Fluorescein Avidin DCS prepared as described in M.O.M. kit instructions. Incubate sections for 5 min *(Please see Note 4)*.
13. Wash sections 2 x 5 min in buffer.

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14. Avidin/biotin blocking step. Perform Avidin/Biotin blocking according to step 4. (This step must be done to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents)
15. Mouse Ig blocking step. Incubate sections for 1 h in working solution of M.O.M. Mouse Ig Blocking Reagent.
16. Wash sections 2 x 2 min in buffer.
17. Protein blocking step. Incubate sections for 5 min in working solution of M.O.M. diluent.
18. Primary antibody. Tip off excess M.O.M. diluent from sections. Dilute second primary antibody in M.O.M. diluent to the appropriate concentration. Incubate section for 30 min *(Please see Note 3)*.
19. Wash sections 2 x 2 min in buffer.
20. Secondary antibody. Apply working solution of M.O.M. Biotinylated Anti-Mouse IgG Reagent. Incubate sections for 10 min.
21. Wash sections 2 x 2 min in buffer.
22. Avidin conjugate. Apply Texas Red™ Avidin DCS at a concentration of 15-20 µg/ml in buffer. Incubate sections for 5–10 min *(Please see Note 4)*.
23. Wash sections for 2 x 5 min in buffer.
24. Mount with appropriate VECTASHIELD® mounting media.

Notes:

1. Aldehyde-fixed tissues (e.g. formalin) tend to be autofluorescent and may make interpretation of specific fluorescein signal difficult.
2. For non-murine tissue, omit step 5 and step 6.
3. Optimal results with the M.O.M. kit are usually obtained with a primary antibody incubation of 30 min. Primary antibody concentrations should be optimized for multiple labeling applications.
4. Optimal order of the fluorescent label should be determined. Other fluorochrome conjugated streptavidin or avidin reagents can be substituted once optimal signal/noise has been established.
5. M.O.M. Troubleshooting Guide is available on our website.

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